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Herpesvirus saimiri

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Herpesvirus saimiri (saimiriine herpesvirus 2) is the classical prototype of the γ_2-herpesviruses or rhadinoviruses, which also contains a human member, the Kaposi's sarcoma-associated herpesvirus. The T-lymphotropic *Herpesvirus saimiri* establishes specific replicative and persistent conditions in different primate host species. Virtually all squirrel monkeys (*Saimiri sciureus*) are persistently infected with this virus. In its natural host, the virus does not cause disease, whereas it induces fatal acute T-cell lymphoma in other monkey species after experimental infection. The virus can be isolated by cocultivation of permissive epithelial cells with peripheral blood cells from naturally infected squirrel monkeys and from susceptible New World monkeys during the virus-induced disease. Tumour-derived and *in vitro*-transformed T-cell lines from New World monkeys release virus particles. *Herpesvirus ateles* is a closely related virus of spider monkeys (*Ateles* spp.) and has similar pathogenic properties to *Herpesvirus saimiri* in other New World primate species.

Similar to other rhadinoviruses, the genome of *Herpesvirus saimiri* harbours a series of virus genes with pronounced homology to cellular counterparts including a D-type cyclin, a G-protein-coupled receptor, an interleukin-17, a superantigen homologue, and several inhibitors of the complement cascade and of different apoptosis pathways. Preserved function has been demonstrated for most of the homologues of cellular proteins. These viral functions are mostly dispensable for the transforming and pathogenic capability of the virus. However, they are considered relevant for the apathogenic persistence of *Herpesvirus saimiri* in its natural host. A terminal region of the non-repetitive coding part of the virus genome is essential for pathogenicity and T-cell transformation. Based on the pathogenic phenotypes and the different alleles of this variable region, the virus strains have been assigned to three subgroups, termed A, B and C. In the highly oncogenic subgroup C strains, the two virus genes *stpC* and *tip* are transcribed from one bicistronic mRNA and are essential for transformation and leukaemia induction. *stpC* fulfils the typical criteria of an oncogene; its product interacts with Ras and tumour necrosis factor-associated factors and induces mitogen-activated protein kinase and nuclear factor kappa B activation. Tip interacts with the RNA transport factor Tap, with signal transduction and activation of transcription factors, and with the T-cellular tyrosine kinase Lck, which is activated by this interaction and phosphorylates Tip as a substrate.

It is of particular interest that certain subgroup C virus strains such as C488 are capable of transforming human T lymphocytes to stable growth in culture. The transformed human T cells harbour multiple copies of the viral genome in the form of stable, non-integrated episomes. The cells express only a few virus genes and do not produce virus particles. The transformed cells maintain the antigen specificity and many other essential functions of their parental T-cell clones. Based on the preserved functional phenotype of the transformed T cells, *Herpesvirus saimiri* provides useful tools for T-cell immunology, for gene transfer and possibly also for experimental adoptive immunotherapy.

Keywords: *Herpesvirus saimiri*; *Herpesvirus ateles*; rhadinovirus; T cells; T lymphocytes

1. NATURAL OCCURRENCE AND PATHOGENICITY

(a) *Apathogenic persistence of or leukaemogenesis by* Herpesvirus saimiri

The rhadinovirus (γ_2-herpesvirus) *Herpesvirus saimiri* (HVS) is regularly found in squirrel monkeys (*Saimiri sciureus*), which naturally occur in South American rainforests. As natural hosts of HVS, squirrel monkeys are infected via saliva usually within the first two years of life. HVS does not cause disease or tumours and establishes lifelong persistence in this species (Meléndez *et al.* 1968).

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The infection with HVS can lead to distinct results in different monkey species (table 1) (Fleckenstein & Desrosiers 1982). Whereas this virus is apathogenic in its natural host, within less than two months after experimental infection, HVS causes acute peripheral T-cell lymphoma in other New World primate species such as tamarins (*Saguinus* spp.), common marmosets (*Callithrix jacchus*) or owl monkeys (*Aotus trivirgatus*) (Meléndez *et al.* 1969; Wright *et al.* 1976; reviewed in Fleckenstein & Desrosiers 1982). These monkey species are presumably not infected by HVS in the wild. The experimental infection is usually performed intramuscularly or intravenously with a typical dose of approximately 10^6

tissue-culture-infectious doses ml^{-1}. Purified virion DNA was also infectious and oncogenic after intramuscular injection into susceptible primates (Fleckenstein *et al.* 1978*a*). Depending on the pathogenic properties and on the sequence divergence in the terminal non-repetitive genomic region (see §2(b) and §4(a)), HVS strains were classified into the three subgroups A, B and C (Desrosiers & Falk 1982; Medveczky *et al.* 1984, 1989). The major representative strains are A11 (Falk *et al.* 1972*a*) for subgroup A, B-S295C (Meléndez *et al.* 1968) and B-SMHI (Daniel *et al.* 1976*a*) for subgroup B, and C488 (Biesinger *et al.* 1990) and C484 (Medveczky *et al.* 1984) for subgroup C. Generally, viruses of subgroup B have the weakest and subgroup C strains the strongest oncogenic properties. Tamarins are most susceptible to viruses of all subgroups, whereas subgroup B viruses do not cause disease in adult common marmosets (table 1) (reviewed in detail in Fleckenstein & Desrosiers 1982; Bröker & Fickenscher 1999; Fickenscher & Fleckenstein 1998).

The pathogenicity of viral deletion mutants based on HVS C488 has been investigated either in common marmosets or in cottontop tamarins (*Saguinus oedipus*) in which the parental strain causes acute peripheral T-cell lymphoma within only a few weeks (Duboise *et al.* 1998*a*,*b*; Knappe *et al.* 1998*a*,*b*). Virus strain C488 induced a similar fulminant disease even in Old World monkeys (*Macaca mulatta*, rhesus monkeys; *Macaca fascicularis*, cynomolgus monkeys) within a few weeks of experimental infection with a high virus dose (Alexander *et al.* 1997; Knappe *et al.* 2000*a*). The pathological findings in these macaques were similar to those in New World primates (Hunt *et al.* 1972; Knappe *et al.* 1998*b*, 2000*a*). The lymphomas in cynomolgus monkeys showed widespread infiltrates consisting of medium-sized and large blasts with vesicular nuclei and prominent nucleoli. The tumour cells were positive for the T-cell marker CD3, but negative for CD20. The infiltrates of blasts involved lymphatic organs such as lymph nodes, spleen and Waldeyer's ring, and other organs including intestine, kidney, liver, lung, pancreas and salivary glands. The lymph nodes were enlarged and showed a widening of sinuses and T zones by the infiltrating blasts as well as a loss of follicular structure. Based on the histopathology, distribution and acute onset, the infiltrates could be designated either as a pleomorphic peripheral T-cell lymphoma or alternatively as a pleomorphic T-lymphoproliferative disorder, showing similarities to human Epstein–Barr virus (EBV)-induced post-transplantation B-lymphoproliferative disease (Knappe *et al.* 2000*a*).

As with the asymptomatic persistent infection in squirrel monkeys, HVS can be isolated from the peripheral blood cells of leukaemic animals, presumably from infected T cells, by co-cultivation with permissive owl monkey kidney (OMK) cells (Daniel *et al.* 1976*b*; Falk *et al.* 1972*a*; Wright *et al.* 1976). HVS replicates productively in the OMK cells and induces cell lysis after 3–20 days. It reaches titres of 10^9 physical particles ml^{-1} and of 10^6–10^7 plaque forming units ml^{-1} and is relatively stable when stored at $+4\,^{\circ}C$ (Fickenscher & Fleckenstein 1998). The virus presents as typical herpesvirus particles, which can be nicely demonstrated by electron microscopy in the cytoplasm of permissive cells (figure 1).

(b) *Semi-permissive persistence in transformed monkey T-cell lines*

A limited series of transformed T-cell lines are available which were derived in the very early days of HVS research from leukaemias or tumours of subgroup A or B virus-infected tamarins (reviewed in Fleckenstein & Desrosiers 1982). Such cell lines have been cultivated continuously for many years. While these cultures initially produced virus particles in most cases, the virus production was lost after prolonged culture. For example, the cell lines 1670 (Marczynska *et al.* 1973; Fleckenstein & Desrosiers 1982) and 70N2 (Falk *et al.* 1972*b*) are derived from virus strain A11, and L77/5 from B-S295C (Fleckenstein *et al.* 1977). Some of these cell lines carried genomic rearrangements or large deletions in the episomal HVS genomes (Kaschka-Dierich *et al.* 1982). Moreover, the episomal DNA is heavily methylated in such cell lines (Desrosiers *et al.* 1979). *In vitro*, marmoset and tamarin T cells can be transformed by HVS to stable growth (Chou *et al.* 1995; Desrosiers *et al.* 1986; Kiyotaki *et al.* 1986; Schirm *et al.* 1984; Szomolanyi *et al.* 1987). These transformed T-cell lines are semi-permissive: they become transformed while still releasing virus particles. In contrast to other viruses such as EBV, there is no indication for a spontaneous switch of a small subpopulation to the lytic cycle in semi-permissive HVS-transformed T cells, in which a broad spectrum of virus transcripts is easily detectable by Northern hybridization (Fickenscher *et al.* 1996*a*). Specifically HVS subgroup C strains such as C488 are able to transform human T cells to stable growth *in vitro* (Biesinger *et al.* 1992). In contrast to semi-permissive T-cell lines from New World monkeys, the C488-transformed human T cells do not produce virus particles (Biesinger *et al.* 1992; Fickenscher *et al.* 1996*a*, 1997). In macaques, which are, as Old World monkeys, more closely related to humans, a rather weak but reproducibly detectable (semi)permissivity of C488-transformed T cells has been described (Alexander *et al.* 1997; Knappe *et al.* 2000*a*). Whereas HVS pathogenicity has not been reported in rodents, a non-permissive infection and tumour induction was described in New Zealand white rabbits, although with variable efficiency (Ablashi *et al.* 1985; Medveczky *et al.* 1989). The species-specific molecular determinants for the cellular susceptibility to permissive or persistent infection are not known. As described, the features of infection can vary considerably between closely related species (e.g. productivity of C488-transformed T cells from macaques versus humans). Not even the macaque model seems optimal for the non-permissive persistence conditions in human T cells (Knappe *et al.* 2000*a*). Thus, it is difficult to predict the behaviour of HVS in another species.

(c) *The closely related* Herpesvirus ateles

A closely related rhadinovirus *Herpesvirus ateles* (HVA), can be isolated at a high rate from spider monkeys (*Ateles* spp.) (reviewed in Fleckenstein & Desrosiers 1982). Isolate no. 810 from *Ateles geoffroyii* (Meléndez *et al.* 1972) is officially classified as ateline herpesvirus type 2, whereas isolate no. 73 and related strains (no. 87, 93, 94) from *Ateles paniscus* are designated as ateline herpesvirus type 3 (Falk *et al.* 1974). HVA replicates in OMK cells (Daniel *et al.* 1976*b*), but remains strictly cell-associated with

Table 1. *Species- and subgroup-specific infection with* HVS

(Minus and plus signs denote negative and positive infection, respectively; perm., permissive; nt, not tested.)

		New World primates			Old World primates		rabbits (New
	HVS subgroup	squirrel monkey	cotton-top tamarin	common marmoset	macaque	human	Zealand white)
peripheral blood cells	A	productive	productive	productive	−	nt	−
	B	productive	productive	−	−	nt	−
	C	productive	productive	productive	productive	nt	non-perm.
T-cell transformation	A	−	+ /semi-perm.	+ /semi-perm.	−	−	−
in vitro	B	−	+ /semi-perm.	−	−	−	−
	C	−	+ /semi-perm.	+ /semi-perm.	+ /semi-perm.	+ /non-perm.	+ /non-perm.
T-cell lymphoma	A	apathogenic	+	+	−	nt	−
	B	apathogenic	+	−	−	nt	−
	C	apathogenic	+	+	+	nt	−

syncytia formation. As a result, supernatants of such cultures have low and unstable HVA titres. Like HVS, HVA is not pathogenic in its natural host, but causes acute T-cell lymphoma in various New World primate species including cotton-top tamarins and owl monkeys (Hunt *et al.* 1972). The pathological changes are similar to those observed after HVS infection. In addition, HVA transforms T cells of certain New World monkey species such as cotton-top tamarins in culture, yielding cytotoxic T-cell lines (Falk *et al.* 1978; Johnson & Jondal 1981*a*,*b*; Kiyotaki *et al.* 1988; reviewed in Fleckenstein & Desrosiers 1982). Human T cells have not been susceptible to transformation with various HVA strains. In comparison with HVS there is far less knowledge about HVA biology.

2. GENOME STRUCTURE AND REPLICATION

(a) *Genome organization of rhadinoviruses*

As a member of the γ-herpesvirus family, HVS is the classical prototype of the subfamily rhadinoviruses (γ_2-herpesviruses) (Albrecht *et al.* 1992*a*; Roizman *et al.* 1992). The human herpesvirus type 8 (HHV-8), which is also termed Kaposi's sarcoma-associated herpesvirus (KSHV) and which will henceforth be referred to as HHV-8/KSHV, is a more recently described member of the rhadinoviruses (Chang *et al.* 1994; Russo *et al.* 1996). HVA has recently been confirmed by sequencing to have a similar genome to HVS (Albrecht 2000; Fleckenstein *et al.* 1978*b*). The genome structures of HVS, HVA, and KSHV/HHV-8 are compared in figure 2. The rhadinoviruses have a so-called M genome with intermediate density in CsCl gradients (M-DNA). The γ_2-herpesviruses were termed 'rhadino' viruses using the ancient Greek word for fragile (Roizman *et al.* 1992), because the M-DNA splits into two DNA molecules of highly different density, the L-DNA containing all the virus genes (low density, low G + C content) and the terminal repetitive H-DNA (high density, high G + C content) without coding capacity. In the case of HVS A11, the H-DNA contains multiple tandem repeats of 1444 bp with 70.8% G + C and a density of 1.729 g ml^{-1}, whereas the long unique L-DNA has 112 930 bp with 34.5% G + C and a density of 1.695 g ml^{-1} (Albrecht *et al.* 1992*a*; Fleckenstein & Desrosiers 1982). The size of the total M-DNA (1.705 g ml^{-1}) genome of approximately 140 000 bp is variable due to the varying copy numbers of H-DNA segments attached to both ends of the linear virion genome.

(b) *Conserved and divergent genome regions in HVS*

The HVS L-DNA genome carries up to 77 open reading frames (Albrecht *et al.* 1992*a*; Albrecht 2000; Biesinger *et al.* 1990; Ensser *et al.* 1997). There are gene blocks of typical herpesvirus genes which are highly conserved also in viruses of other herpesvirus families (Albrecht & Fleckenstein 1990; Gompels *et al.* 1988; Nicholas *et al.* 1992*a*). In addition there are blocks of genes which do not usually occur in other herpesviruses. Among these genes are transforming oncogenes and a series of viral homologues of cellular genes which will be described below. While most genes are well conserved between different HVS strains (Knappe *et al.* 1997), there is extensive sequence variation at the so-called left end of the L-DNA. Together with the different pathogenicity in different hosts (table 1), this divergence formed the basis for the classification of HVS strains into the subgroups A, B and C (Medveczky *et al.* 1984, 1989; Szomolanyi *et al.* 1987). Deletion mutants of this region lose their transforming potential (Desrosiers *et al.* 1985*a*, 1986; Duboise *et al.* 1996, 1998*b*; Knappe *et al.* 1997; Medveczky *et al.* 1993). Whereas virus strains of all subgroups were capable of inducing T-cell lymphoma in susceptible non-human primates, only subgroup C strains of HVS were able to transform human T cells *in vitro* (Biesinger *et al.* 1992). The transformation-relevant genes encoded in the respective left-terminal L-DNA region and the functions of the derived gene products will be described below (§ 4). In addition, the region of the R transactivator gene *orf50* occurs as two strongly divergent alleles in the strains A11 and C488 (Thurau *et al.* 2000).

(c) *Viral functions for lytic replication and episomal persistence*

Not much is known about the replication mechanisms of rhadinoviruses in general or in particular of HVS. In comparison with the herpesvirus prototype herpes simplex virus, the mechanism is simplified because there is only a single unique region and the terminal repetitions are simple tandem-repeated segments without coding

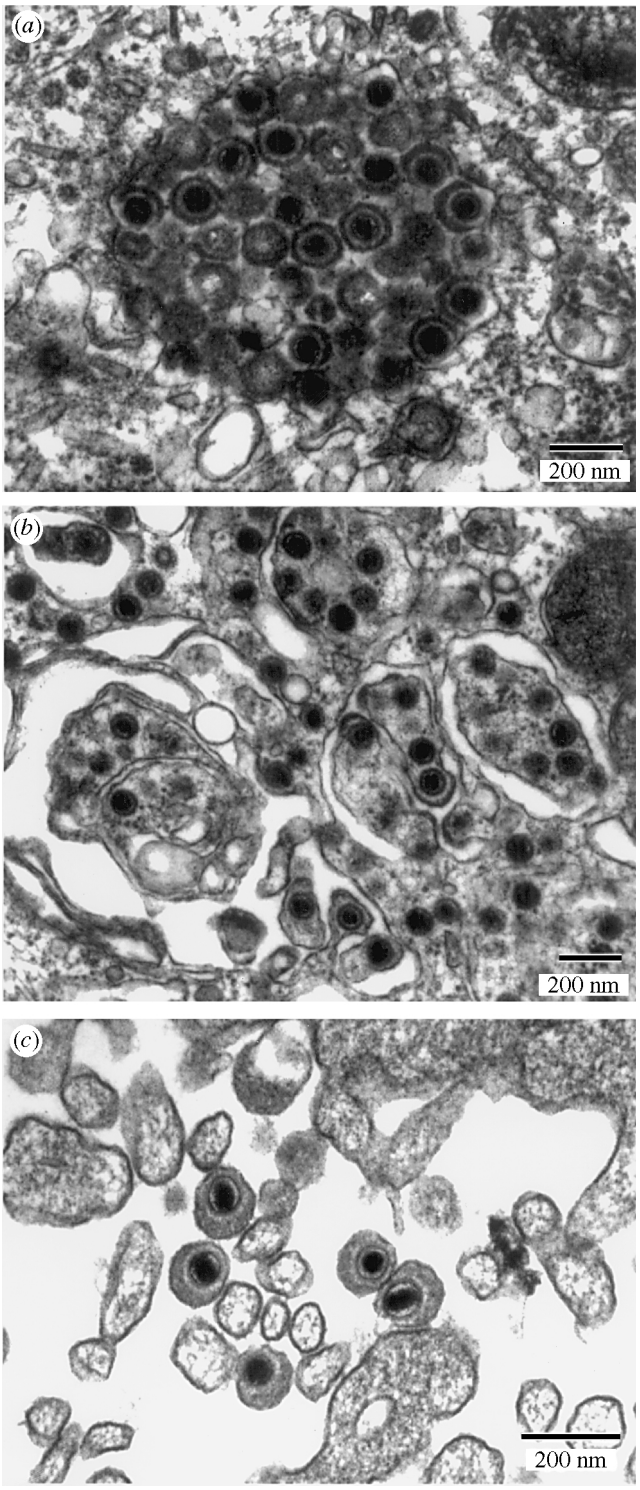

Figure 1. Morphogenesis of HVS A11 in permissive cells. (*a*) Fully enveloped, mature HVS particles and nucleocapsids found within an inclusion body in the cytoplasm of an infected cell. Many of these virus particles are not exhibiting an electron-dense viral core. (*b*) HVS nucleocapsids seen within the cytoplasm of an infected cell. Some of the particles are budding into vacuolated areas of the cell, thus obtaining their exterior viral envelope. (*c*) Fully enveloped, mature extracellular HVS particles with electron-dense viral DNA, internal capsid structure, and exterior envelope. Figures provided by Dr Dharam Ablashi and Dr Bernard Kramarsky (Advanced Biotechnologies Inc., Columbia, MD, USA).

capacity. On the basis of the rolling-circle mechanism for replication, recombinant viruses were generated by homologous recombination into the right-terminal L- to H-DNA transition (Grassmann & Fleckenstein 1989). In this case, a single crossing-over event was sufficient to integrate the recombination plasmid into the viral genome. Because H-DNA segments were not included in this plasmid, it is obvious that the terminal repeats are automatically attached to the right L-DNA terminus during rolling-circle replication. The origin of lytic replication was mapped to the untranslated region upstream of the thymidylate synthase gene (Lang & Fleckenstein 1990; Schofield 1994).

In transformed human T cells, HVS persists as stable non-integrated episomes at high copy number (Biesinger *et al.* 1992). Surprisingly, a new isolate termed C139 was found to persist episomally at low copy number in transformed human T cells (Fickenscher *et al.* 1997). There are no indications yet for the genetic correlate of a plasmid-like origin of replication and of the viral factors involved. By analogy to observations made for KSHV/HHV-8, the *orf73* product was suggested to be involved because it shares homology to the latent nuclear antigen of KSHV/HHV-8. However, this is unlikely, because transcripts of this gene were not detectable by Northern blotting and by subtractive cDNA cloning from C488-transformed human T cells (Fickenscher *et al.* 1996*a*; Knappe *et al.* 1997). A DNA fragment of strain C484 was described to mediate plasmid maintenance (Kung & Medveczky 1996). However, this fragment is from the variable region of the left-terminal L-DNA, which had been previously shown to be dispensable for episomal persistence (Medveczky *et al.* 1989).

(d) *Viral regulatory genes*

In contrast to herpes simplex virus, the infection of tissue culture cells by HVS is asynchronous (Randall *et al.* 1985). Therefore, the classification of HVS genes to the immediate-early (IE) phase of infection has been difficult and contradictory in the past, and was mostly based on experiments using cycloheximide to inhibit protein synthesis. The IE gene *ie57* codes for a nuclear phosphoprotein of 52 kDa (Hoyle *et al.* 1990; Nicholas *et al.* 1988, 1990; Randall *et al.* 1984). This protein shares structural and functional homology with ICP27/IE63 of herpes simplex virus. Correspondingly, IE57 acts as a post-transcriptional regulator which stimulates the expression of unspliced and represses the expression of spliced transcripts (Whitehouse *et al.* 1998*a*). Moreover, IE57 redistributes nuclear components of the splicing machinery (Cooper *et al.* 1999) and is involved in nuclear RNA export (Goodwin *et al.* 1999).

Besides *ie57* only two other genes were assigned to the IE phase. Contradictory results were published about transcripts for the thymidylate synthase gene *orf70*, which have been reported as either detectable or not in the presence of cycloheximide (Bodemer *et al.* 1984, 1986; Nicholas *et al.* 1990). Presumably, the unusual transcription behaviour is related to the fact that the lytic replication origin is localized in the promoter region of this gene (Lang & Fleckenstein 1990; Schofield 1994). Another IE transcript was mapped to the gene *ie14* (Hoyle *et al.* 1990; Nicholas *et al.* 1990). The derived gene product has sequence homology to murine superantigens

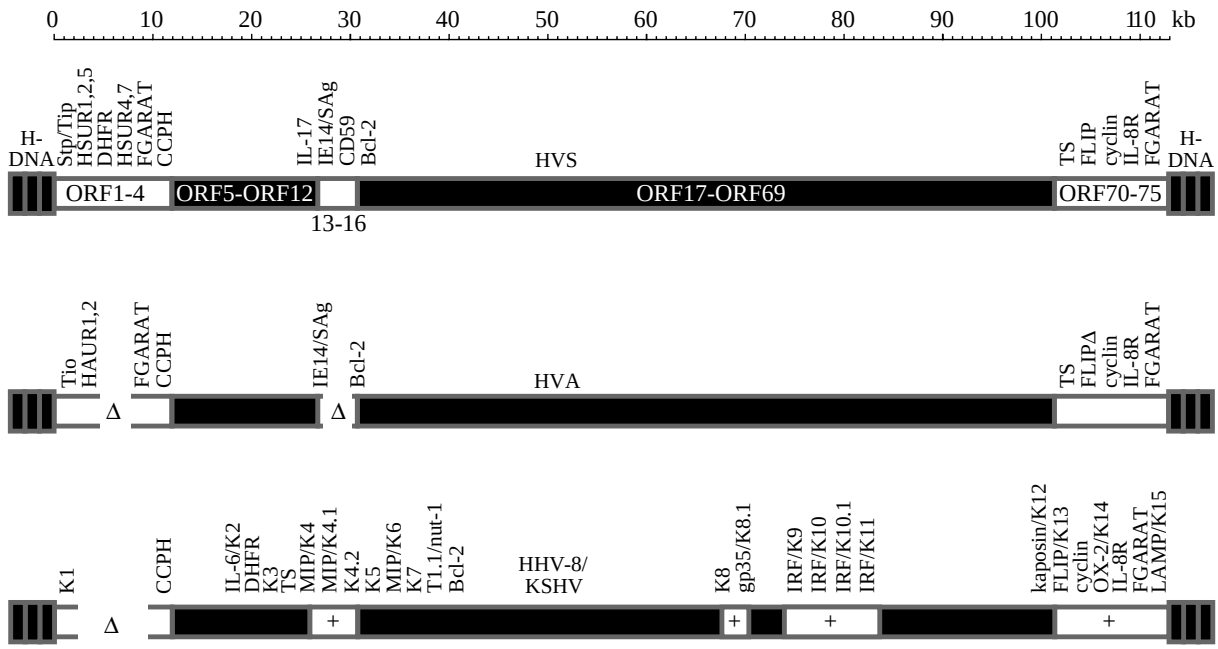

Figure 2. Genome structures of selected rhadinoviruses. For comparison, the genome structures of the rhadinoviruses HVS (Albrecht *et al*. 1992*a*), HVA (Albrecht 2000) and KSHV/HHV-8 (Russo *et al*. 1996) are depicted. Rather conserved genomic regions of virus genes with typical herpesvirus functions are shown in black. As white boxes, the variable areas are marked harbouring genes with homology to cellular counterparts. All these genes are mentioned with the currently used abbreviation in the text. In comparison with HVS, HVA has two deletions in such areas (marked with a delta). In contrast, KSHV/HHV-8 carries a series of further specific genes (marked with a plus sign) many of which have been related to transforming functions.

(Sag) Nicholas *et al*. 1990; Thomson & Nicholas 1991). However, the *ie14*/*sag* open reading frame was shown to be dispensable for virus replication (Duboise *et al*. 1998*a*; Knappe *et al*. 1997, 1998*a*,*b*), which largely excludes a contribution of this gene to the regulation of virus replication. Thus, *ie57* appears to be the sole regulatory viral IE gene.

By co-transfection experiments, a strong viral transactivator function was mapped to the delayed-early *orf50* gene (Nicholas *et al*. 1991). This protein shows a limited sequence homology to the R transactivator of EBV. Due to differential splicing and promoter usage, the gene from HVS A11 codes for a larger protein ORF 50A and for a smaller C-terminal variant ORF 50B (Whitehouse *et al*. 1997*a*). Only ORF 50A strongly transactivated reporters carrying the promoters of responsive virus genes, such as *orf6* and *orf57* (Whitehouse *et al*. 1997*b*, 1998*b*). The C-terminal amino acids of the ORF 50 proteins represented the transactivation domain and were shown to bind to the TATA-binding protein in the basal transcription complex (Hall *et al*. 1999). Surprisingly, the genomic *orf50* region was found to be strongly divergent between the virus strains A11 from subgroup A and C488 from subgroup C (Thurau *et al*. 2000). Moreover, a strong antisense transcription was detected for C488 *orf50* during later phases of lytic replication. In contrast to strain A11, the ORF 50B protein of C488 had full transactivation properties (Thurau *et al*. 2000). Because IE57-mediated post-transcriptional inhibition does not have functional relevance in this context, this suggests that *orf50* exerts the dominant function for replication regulation, at least in HVS strain C488.

3. VIRUS GENES WITH HOMOLOGY TO CELLULAR COUNTERPARTS

(a) *Molecular piracy of cellular genes*

Rhadinoviruses have pirated a series of genes from cellular genomes (figure 2). Remarkably, the viral gene copies are usually not disrupted by intron sequences, which theoretically would imply a role for reverse transcription, possibly involving endogenous retroviruses. Whatever the mechanism, the fact that several of these pirated cellular genes are common to several rhadinoviruses and even to other γ-herpesviruses including EBV, suggests that the uptake of cellular genes is a rare event during evolution. Like other rhadinoviruses, HVS has acquired from its host cells a series of genes which are listed in table 2. The transformation-associated gene products StpC and Tip share limited homology to cellular proteins. Because these genes are required for T-cell transformation and pathogenicity (Duboise *et al*. 1998*b*; Knappe *et al*. 1997), they are described in detail in a separate section (§ 4). Whereas StpC and Tip are constitutively expressed, most of the cell-homologous genes are not transcribed in C488-transformed human T cells, which are non-permissive for virus replication (Fickenscher *et al*. 1996*a*; Knappe *et al*. 1997). Most of these cell-homologous virus genes are in fact expressed during lytic virus replication. Thus, in the natural host, the squirrel monkey, they might play a role either in enhancing lytic replication and spreading, or in ensuring the apathogenic persistence of HVS while evading the host's immune defence mechanisms. Some of the products of the cell-homologous genes exert stimulatory effects on T cells. However, T-cell

Table 2. *HVS genes with homology to cellular counterparts*

(Minus signs denote not essential; nt, not tested.)

virus gene	ORF	cellular homologue	amino-acid identity (%)	functional context	essential for	
					T-cell transformation	pathogenicity
StpC	01b	collagen	local	oncogene	+	+
Tip	01a	Src kinases	local	Lck interaction	+	+
DHFR	02	DHFR	83	nucleotide metabolism	−	nt
5 URNAs		U-RNA, snrp	local	splice regulation (?)	−	nt
FGARAT	03	FGARAT	local	nucleotide metabolism	nt	nt
CCPH	04	C4b binding protein	34	complement inhibition	nt	nt
IL-17	13	IL-17	57	cytokine	−	−
IE14/vSAg	14	Mls and MMTV superantigens	22	cytokine, mitogen	−	+/−
CD59	15	CD59	49	complement inhibition	nt	nt
Bcl-2	16	Bcl-2 homology domains BH1, BH2	local	apoptosis inhibition	nt	nt
TS	70	thymidylate synthase	66	nucleotide metabolism	nt	nt
FLIP	71	FLICE/caspase-8 FADD, c-FLIP	local	apoptosis inhibition	−	−
cyclin	72	cyclin D	25	cell cycle	−	−
IL-8R	74	IL-8R, G-protein-coupled receptors	30	cytokine receptor	−	nt
FGARAT	75	FGARAT	local	nucleotide metabolism	−	nt

transformation or HVS-induced leukaemogenesis has not been reported for the natural host.

(b) *Cell-homologous genes of nucleotide metabolism and cell-cycle control*

A first group of viral proteins are homologous to enzymes of the nucleotide metabolism or to cell-cycle-promoting factors. *Orf2* codes for a dihydrofolate reductase (DHFR) (Ensser *et al.* 1999; Trimble *et al.* 1988) and *orf70* for a functional thymidylate synthase (Bodemer *et al.* 1984, 1986; Honess *et al.* 1986). *Orf2* and the viral U-RNA genes are dispensable for virus replication and T-cell transformation (Ensser *et al.* 1999). Both *orf3* and *orf75* encode large tegument proteins, which share local homology to formylglycineamide ribotide amidotransferase (FGARAT) (Albrecht 2000; Ensser *et al.* 1997). These enzymatic functions may play a role in augmenting the free nucleotide pool and thus facilitate DNA synthesis and virus replication. *Orf72* codes for a functional viral cyclin D version (Jung *et al.* 1994; Nicholas *et al.* 1992*a*,*b*). In contrast to cellular cyclin D, the viral cyclin is not inactivated by the cyclin-dependent kinase inhibitors p16, p21 and p27 (Swanton *et al.* 1997). This deregulation pushes the cell cycle towards the S phase and thereby may support virus replication in permissive cells (Swanton *et al.* 1997). Enhanced replication might secondarily promote transformation or tumour induction. The expression of the viral cyclin in semi-permissive HVS-transformed marmoset T cells might contribute to their activated and transformed phenotype (Jung *et al.* 1994). However, these functions would appear to be auxiliary rather than essential since the viral cyclin has been shown not to be required for replication, T-cell transformation and lymphomagenesis (Ensser *et al.* 2001).

(c) *Viral homologues of complement-regulatory proteins*

Two other cell-homologous HVS genes target the complement system, which is a relevant mechanism for pathogen elimination. *Orf4* codes for a complement-control protein homologue (CCPH), which inhibits C3 convertase. This enzyme is involved in the initiation of early steps in complement activation (Albrecht & Fleckenstein 1992; Fodor *et al.* 1995). *Orf15* is a viral variant of CD59 and blocks the terminal complement cascade (Albrecht *et al.* 1992*b*; Bramley *et al.* 1997; Rother *et al.* 1994). Both proteins may be used for the escape from immune surveillance *in vivo*. In addition, T-cell stimulatory functions have been described for cellular CD59, which can act as a ligand for the CD2 receptor protein on T cells (Deckert *et al.* 1995; Korty *et al.* 1991; Naderi *et al.* 1999). Although functional data are not yet available for the viral counterpart of CD59, a similar T-cell stimulatory function is possible.

(d) *Viral inhibitors of apoptosis*

A third class of lytically expressed viral proteins are potent inhibitors of cell death or apoptosis. The product of *orf71* is a viral FLICE (FADD-like interleukin 1-converting enzyme (ICE)-like protease) inhibitory protein (FLIP) sharing the death-effector domains with FLICE (caspase 8) and cellular FLIP (Thome *et al.* 1997). In various cell types, the viral FLIP inhibited death-receptor-dependent apoptosis mediated by Fas ligand, tumour necrosis factor-α (TNF-α), TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) or TRAMP (tumor necrosis factor receptor-like apoptosis-mediating protein). The viral FLIP was shown to interact with FADD (Fas-associated protein with death domains) and FLICE via homophilic interaction of their death-effector domains. Thus, the

formation of the death-signal-induced signalling complex was blocked, caspase 8 (FLICE) was not activated, and cell death did not occur. Moreover, HVS infection partially protected permissive OMK cells from Fas-dependent apoptosis at a late stage of infection, immediately before the cells are lysed to release the infectious particles (Thome *et al.* 1997). However, the apoptosis-blocking activity of FLIP was dispensable for virus replication, T-cell transformation and lymphoma induction (Glykofrydes *et al.* 2000). The product of *orf16* contains the Bcl-2 homology domains BH1 and BH2 and shows anti-apoptotic activity similar to cellular Bcl-XL. Both Bcl-XL and viral Bcl-2 inhibit cell death induced by either cell-autonomous (independent of death receptors) or receptor-mediated mechanisms, depending on the cell type studied. The viral Bcl-2 homologue was shown to act at the level of mitochondrial stabilization and to inhibit apoptosis induced by Sendai virus infection or by treatment with Fas ligand, dexamethasone or menadione, or by irradiation (Bellows *et al.* 2000; Derfuss *et al.* 1998; Nava *et al.* 1997). Thus, two HVS-encoded proteins appear to render infected cells resistant both to death-receptor-mediated and to cell-autonomous (death-receptor-independent) apoptosis mechanisms, resulting in enhanced virion production from permissive cells (Meinl *et al.* 1998).

(e) *Viral cytokine-related genes*

A fourth group of cell-homologous viral genes is related to the cytokine network. This includes viral homologues of the cytokine interleukin-17 (IL-17) and of the IL-8 chemokine receptor. The HVS gene *orf13* led to the discovery of its cellular homologue, which was initially termed *ctla8* (Rouvier *et al.* 1993). This gene codes for IL-17, a cytokine produced specifically by $CD4^+$ T cells. IL-17 induces the secretion of IL-6, IL-8, G-CSF (granulocyte colony stimulating factor), and PG-E2 (prostaglandin E2) by stroma cells such as fibroblasts, endothelial or epithelial cells, and promotes the proliferation and maturation of $CD34^+$ haemopoietic progenitor cells into neutrophilic granulocytes. Among various other functions, IL-17 was shown to be capable of supporting T-cell proliferation (Fossiez *et al.* 1996, 1998; Kennedy *et al.* 1996; Spriggs 1997; Yao *et al.* 1995*a*,*b*, 1996*a*). The viral IL-17 is functionally not distinguishable from its cellular counterpart. Viral null mutants for *orf13*/*IL-17* had full replicative, transforming and pathogenetic capabilities (Knappe *et al.* 1998*a*). The product of *orf74* is a viral IL-8 receptor (IL-8R). It is a seven transmembrane G-protein-coupled receptor classified to the low-affinity B type of IL-8R (Ahuja & Murphy 1993; Murphy 1994; Nicholas *et al.* 1992*b*). A paracrine stimulation model was postulated for *in vivo* virus replication, whereby viral IL-17, produced by virus-infected cells, would induce IL-8 on neighbouring stroma cells. This IL-8 might then bind to the viral IL-8R on the virus-infected cell and lead to further activation. However, the existence of such a paracrine stimulation mechanism has not yet been substantiated by experimental data.

(f) *The viral homologue to murine superantigens*

Finally, *orf14* was one of the few viral IE genes which were assumed to play a role in the regulation of virus replication (Nicholas *et al.* 1990). A protein sequence homology was noted to the superantigen (Sag) of mouse mammary tumour virus (MMTV) and to murine *mls* superantigens, leading to the designation IE14/vSag for the *orf14* product. Superantigens are characterized by their highly efficient antigen-independent stimulation of T cells if they express the superantigen-specific Vβ families of T-cell receptor molecules. Superantigens cross-link the Vβ chains of the T-cell receptor to major histocompatibility complex (MHC) class II molecules on accessory cells, leading to T-cell stimulation. The recombinant viral IE14/vSag bound to MHC class II molecules and stimulated T-cell proliferation. However, there is as yet no evidence for a selective advantage for certain Vβ families, neither after stimulation of human T cells with IE14/vSag *in vitro*, nor after infection and transformation with HVS (Duboise *et al.* 1998*a*; Knappe *et al.* 1997; Yao *et al.* 1996*b*). Therefore, the *ie14* product should be designated either 'superantigen homologue' or simply 'mitogen'. The deletion of *ie14*/*vsag* from HVS C488 did not impair its ability to induce tumours in cotton-top tamarins, nor to transform human and simian T cells *in vitro* (Knappe *et al.* 1997, 1998*b*). In another study, however, similar deletion mutants did not induce T-cell lymphoma in common marmosets and they were unable to transform T cells from this species *in vitro*. Surprisingly, this virus did not even persist in infected animals (Duboise *et al.* 1998*a*). This contradiction could be explained either by species-specific differences between New World primate species, or by comparably lower virus titres used for the experimental infection in the latter study.

It is possible that *ie14*/*vsag* plays a role in the apathogenic persistence of HVS in the squirrel monkey, the natural host of the virus (Knappe *et al.* 1997, 1998*a*,*b*). A similar situation as known for the superantigen from MMTV in mice is conceivable. In this case, the superantigen facilitates infection and spread of the virus in B cells through attracting and stimulating T helper cells, followed later by apoptosis and specific deletion of the superantigen-stimulated lymphocytes from the T-cell repertoire (Held *et al.* 1993, 1994).

(g) *General role for the cell-homologous genes of HVS*

In summary, leaving aside *stpC* and *tip,* there is little evidence for a functional role of the cell-homologous genes of HVS in the transformation of human T cells. Growing knowledge from deletion mutagenesis studies demonstrates that most if not all of these genes are dispensable for virus replication, T-cell transformation in culture, and pathogenesis in susceptible New World primates (table 2). However, the situation may be different in New World monkeys, where HVS establishes a (semi)permissive infection. Under these conditions, most cell-homologous genes are transcribed (Fickenscher *et al.* 1996*a*), and additional proteins such as the viral cyclin could contribute to the transforming and pathogenic phenotype of HVS. The essential genes involved in T-cell transformation are *stpC* and *tip* as described below (§ 4). By contrast, the pirated cell-homologous genes are most probably involved in the efficient infection, replication and spreading, and possibly in the apathogenic persistence of HVS in its natural host, the squirrel monkey.

4. TRANSFORMING FUNCTIONS

(a) *The transformation-associated genomic region of HVS*

The induction of T-cell leukaemia *in vivo* and the observation of T-cell transformation *in vitro* raised questions as to the molecular mechanisms involved on the viral and the cellular side. Early deletion experiments attributed the essential role for transformation and pathogenicity to the variable region at the left end of the HVS L-DNA (Desrosiers *et al.* 1985*a*, 1986; Koomey *et al.* 1984; Murthy *et al.* 1989). In subgroup A strains there is only one gene at this position, termed *stpA* (saimiri transformation-associated protein of subgroup A strains) (Murthy *et al.* 1989). The position-homologous saimiri transformation-associated protein of subgroup B strains (*stpB*) lacked transforming activity (Choi *et al.* 2000). At the homologous location, the virus strains of subgroup C carry two open reading frames (Biesinger *et al.* 1990), which were later termed *stpC* (saimiri transformation-associated protein of subgroup C strains) and *tip* (tyrosine kinase interacting protein) (Biesinger *et al.* 1990, 1995; Jung & Desrosiers 1991). StpA shares limited sequence homology with StpC, but is structurally unrelated to Tip. The deletion of any of these genes abolishes the transformation capacity of HVS *in vivo* and *in vitro*, while none of them is required for viral replication (Duboise *et al.* 1996, 1998*b*; Knappe *et al.* 1997; Medveczky *et al.* 1993). The closely related HVA encodes the protein Tio at the homologous genomic position (Albrecht *et al.* 1999). All these transformation-related proteins of HVS and HVA carry a hydrophobic C-terminal membrane-anchor sequence. Current knowledge about the molecular mechanisms of the transforming functions of HVS and HVA is schematically depicted in figure 3.

(b) *The saimiri transformation-associated protein Stp*

The *stpC* gene product is a small perinuclear membrane-associated phosphoprotein with a predicted molecular mass of approximately 10 kDa and an apparent molecular mass of 20 kDa. The N-terminus of StpC consists of 17 mostly charged amino acids. The C-terminus is a hydrophobic region, which probably serves as an anchor to perinuclear membranes. In between, there are approximately 17 collagen tripeptide repeats $(GPX)_n$, which may mediate trimerization of the protein (Biesinger *et al.* 1990; Fickenscher *et al.* 1997; Jung & Desrosiers 1991, 1992, 1994). StpA of strain A11 and StpC of strain C488 are oncoproteins: transfected rodent fibroblasts formed foci *in vitro* and induced tumours in nude mice (Jung *et al.* 1991). *stpA* transgenic mice developed polyclonal peripheral T-cell lymphoma, while an *stpC* transgene induced various epithelial tumours (Kretschmer *et al.* 1996; Murphy *et al.* 1994). StpA was reported to bind to and to be phosphorylated by the non-receptor tyrosine kinase Src (figure 3) (Lee *et al.* 1997). The non-transforming StpB also associates with Src (Choi *et al.* 2000). StpC was shown to interact with the small G protein Ras favouring its active GTP-bound state and stimulating mitogen activated protein (MAP) kinase activity (figure 3) (Jung & Desrosiers 1995). Both proteins StpA and StpC interact with TNF-associated factors (TRAFs) leading to nuclear factor kappa B (NF-κB) activation (Lee *et al.* 1999). Although the precise biochemical mechanisms still have to be resolved, the transforming potential of StpA and StpC is well established.

(c) *The tyrosine-kinase interacting protein Tip*

The function of Tip, which only exists in subgroup C strains, has been investigated intensively, but there is still no consensus view of its function. Conflicting observations exist and three different molecular associations have been described (figure 3). The open reading frame *tip* is situated downstream of *stpC* at the left-terminal genomic end of the L-DNA. Both *stpC* and *tip* are transcribed as a single bicistronic mRNA from a common promoter complex. The transcription of *stpC*/*tip* is regulated similarly to cellular IE genes in T cells and is not obviously dependent on other viral factors. In C488-transformed human T cells, StpC and Tip are the only viral proteins which have been shown to be constitutively expressed (Biesinger *et al.* 1995; Fickenscher *et al.* 1996*a*, 1997; Knappe *et al.* 1997). This is consistent with a simple model for the transformation of human T cells by HVS that is further supported by the observation that either *stpC* or *tip* or both together are essential for transformation and pathogenicity (Duboise *et al.* 1998*b*; Knappe *et al.* 1997). In contrast, the situation in semi-permissive simian T cells is more complex, because a broad range of viral genes is expressed (Fickenscher *et al.* 1996*a*).

The first hint of the molecular function of Tip came from the observation of a 40 kDa phosphoprotein which was associated with the T-cell-specific non-receptor tyrosine kinase $p56^{lck}$ in C488-transformed T cells as demonstrated by immunoprecipitation with Lck antibodies and phosphotransferase assay. The protein could be identified as the gene product of the leftmost C488 reading frame, which was therefore called tyrosine kinase interacting protein (Tip) (Biesinger *et al.* 1995). Tip of HVS strain C488 has a predicted molecular mass of 29 kDa with an apparent size of 40 kDa. The Tip proteins carry an N-terminal glutamate-rich region, which is duplicated in some strains, one or two serine-rich regions, and a C-terminal hydrophobic domain which anchors the molecule at the inside of the plasma membrane (Biesinger *et al.* 1990; Fickenscher *et al.* 1997; Lund *et al.* 1995, 1996). Only five amino acids are assumed to project into the extracellular space (Lund *et al.* 1996). The molecule contains several tyrosine residues, three of which are conserved between all strains investigated. These tyrosines are a substrate for Lck, a tyrosine kinase which plays a central role in T-cell development and activation. The association between Tip and Lck is mediated by two sequence motifs, which are located in the C-terminal third of Tip: the C-terminal Src kinase homology domain (CSKH) of nine amino acids, which is similar to the regulatory regions of various Src kinases, and a proline-rich SH3-domain-binding sequence. Both motifs are required for the interaction with the kinase (Biesinger *et al.* 1995; Jung *et al.* 1995*a*).

Several groups have shown that the binding of herpesviral Tip to the tyrosine kinase Lck results in a strong activation of the enzyme (Fickenscher *et al.* 1997; Hartley *et al.* 1999; Lund *et al.* 1997*a*; Wiese *et al.* 1996). This was observed by different experimental approaches using

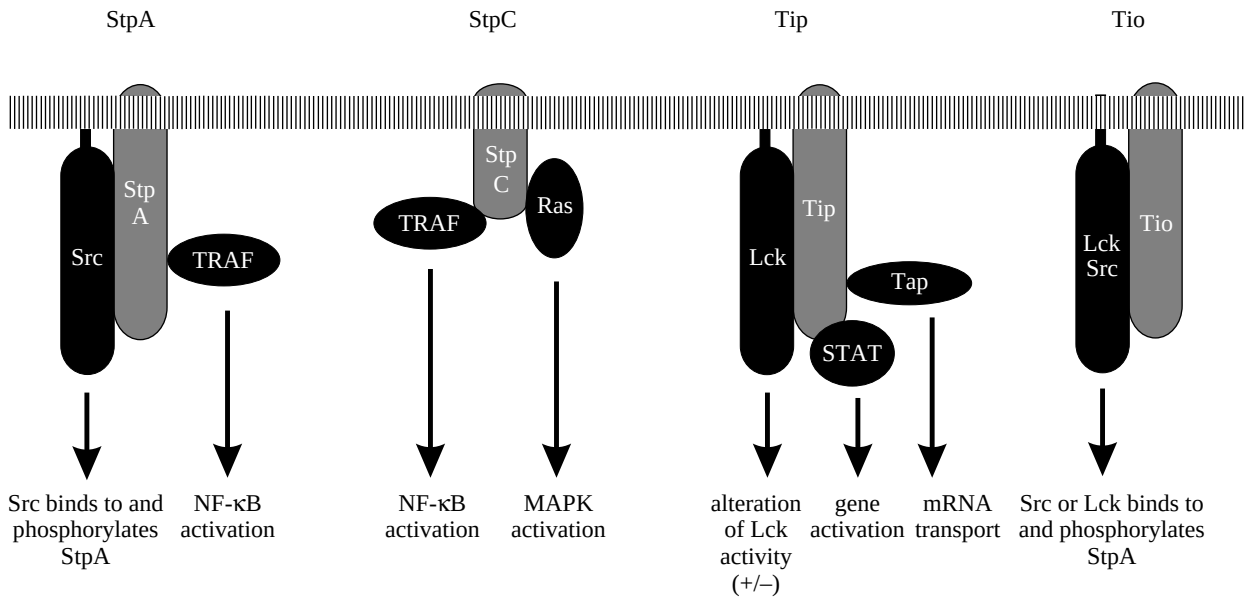

Figure 3. The transformation-associated proteins of HVS and HVA. The transformation-associated proteins StpA (HVS A11), StpC, Tip (HVS C488) and Tio (HVA) are schematically depicted together with cellular proteins which have been shown to interact with these proteins. The functional effects of these interactions are given. All four viral proteins have a similar C-terminal hydrophobic sequence for membrane association.

native as well as recombinant molecules, transfected cells or cell-free systems. Recombinant Lck was activated by recombinant Tip *in vitro*, showing that their interaction is direct and does not depend on other T-cell molecules (Wiese *et al.* 1996). The interaction between Lck and Tip is highly selective: the other Src family members, which are active in transformed T cells, $p60^{fyn}$ and $p53/56^{lyn}$, did not phosphorylate Tip, nor were they activated by Tip (Fickenscher *et al.* 1997; Wiese *et al.* 1996). Lck was found to be activated by Tip even if the regulatory tyrosines Y394 and Y505 of Lck had been mutated, suggesting a novel mechanism of Lck activation (Hartley *et al.* 1999). Lck has a central role in T-cell signalling, and constitutively active Lck mutants have an oncogenic potential. This suggests that the herpesvirus-induced activation of Lck signalling contributes to the activated phenotype of HVS-transformed T cells and to the transformation process. According to this hypothesis, Tip and StpC would act in synergy.

However, contradictory results were obtained by stable transfection of *tip* into Jurkat T cells and fibroblasts. Jurkat T cells with high levels of Tip expression had low basal levels of tyrosine phosphorylation, and their response to T-cell-receptor activation was impaired (Jung *et al.* 1995*b*). This effect was even more pronounced when Tip was mutated in position Y114 to enhance its binding to Lck (Guo *et al.* 1997). In addition, Tip partially reversed the transformed phenotype of fibroblasts which had lost contact inhibition after transfection with a constitutively active mutant of Lck (Guo *et al.* 1997; Jung *et al.* 1995*b*). Consequently, an alternative model assigned to Tip a different role: the function of Tip in T-cell transformation by HVS seems comparable with that of latent membrane protein (LMP)-2A in EBV infection. LMP-2A inhibits B-cell-receptor-mediated signalling in EBV-transformed B cells and favours the latent form of infection by blocking EBV replication (Brielmeier *et al.* 1996; Fruehling & Longnecker 1997; Miller *et al.* 1994). This alternative hypothesis suggests that Tip is a functional antagonist of StpC, attributing to Tip an inhibitory role. It is conceivable that the activation of Lck and the inhibition of T-cell signalling by Tip are two different aspects of the same function if one postulates that the herpesvirus activation of Lck by Tip might trigger negative feedback mechanisms, especially in stably transfected Jurkat cells. Since T-cell signalling is a complex regulatory process, it is perhaps not surprising that conflicting effects of herpesvirus Tip have been observed under different experimental conditions.

It is difficult to decide which aspects of Tip function are important in the transformation of primary human T cells with C488. The reason for this is the very low expression levels of Tip in transformed human T cells. Tip cannot be demonstrated by Western blotting; the more sensitive immune complex kinase assay after Lck immunoprecipitation is required for this purpose. On the other hand, $p56^{lck}$ is an abundant molecule and any changes in its enzymatic activity which might be caused by binding of Tip may be masked by the excess free Lck. In an early study, no alteration in the expression and activity of Lck was observed after transformation of T cells (Bröker *et al.* 1994). A constitutive activation of Lck in HVS-transformed T cells has been described, but only in comparison with human T-cell leukaemia virus type 1 (HTLV-1)-transformed T-cell lines (Noraz *et al.* 1998). Another feature of transformed human T cells argues in favour of a synergism between Tip and StpC: when compared with their non-infected parental clones, HVS C488-transformed human T-cell clones had increased basal levels of tyrosine phosphorylation (Wiese *et al.* 1996).

Further results have raised doubts about the relevance of the Tip–Lck interaction for the transformation process.

It is obvious that Tip is necessary for the transformation of human and common marmoset T cells by subgroup C strains *in vitro* and for tumour formation in infected common marmosets *in vivo* (Duboise *et al.* 1996, 1998*b*; Knappe *et al.* 1997; Medveczky *et al.* 1993). However, the transforming function of Tip can probably be separated from its binding to Lck. A Tip mutant deficient for the SH3-binding motif was unable to associate with Lck, yet the respective C488 mutant was still able to transform simian T cells *in vitro*, and even induced tumours in these animals (Duboise *et al.* 1998*c*). Although it is presently impossible to draw firm conclusions on the relevance of the Tip–Lck interaction, the question should be seen in the context of the natural infection of squirrel monkeys in which T-cell transformation obviously does not occur. The viral functions allowing the Tip to Lck interaction must have been developed in the natural host, and a better understanding of HVS biology in squirrel monkeys could help to elucidate this problem.

Besides its interaction with Lck, Tip has been shown to associate with signal transducer and activator of transcription (STAT) factors. An activation of STAT1 and STAT3 had been initially observed after transfection of Tip-C484 into Jurkat T cells (Lund *et al.* 1997*b*). The STAT factors were found complexed with Lck and Tip, and STAT3 was phosphorylated in these immunoprecipitates (Lund *et al.* 1997*b*). The 48 amino-acid Lck-binding domain of Tip, including the CSKH and SH3B domains, was sufficient for activation of Lck and STAT3 in Jurkat cells (Lund *et al.* 1999). In addition, tyrosine phosphorylation (Y72; identical to Y114 of C488) in the N-terminal portion of C484 Tip by Lck was required for STAT factor binding and activation of STAT factor-dependent transcription (Hartley & Cooper 2000). The respective region YXPQ of Tip is a consensus binding site for STAT factors. These observations further support the first model for Tip function, which is based on Lck activation by Tip.

A third cellular Tip-interacting factor, termed Tap (Tip-associated protein), was identified by a yeast two hybrid screen using a C-terminal deletion mutant of Tip harbouring amino acids 1–226 as a bait, thus including the SH3B domain (Yoon *et al.* 1997). Tap, a protein of 65 kDa, is an RNA export factor (Grüter *et al.* 1998), for which no T-cell-specific function is as yet known. Coexpression of Tip and Tap resulted in strong aggregation of Jurkat T-leukaemia cells in parallel with overexpression of adhesion molecules and NF-κB activation. Transfection of *tap* into Tip-expressing Jurkat cells reversed the signalling inhibition caused by *tip* alone (Yoon *et al.* 1997). The role of Tap in the transformation of primary simian or human T cells has not yet been investigated.

(d) *The HVA gene* tio *from the transformation-associated genome region*

In HVA strain 73, a split gene was detected in the left-terminal L- to H-DNA transition region. The derived viral protein, termed Tio ('two in one'), shares local sequence homology with StpC and Tip of HVS. Tio is expressed in HVA-transformed simian T cells. After co-transfection, Tio bound to and was phosphorylated by the Src kinases Lck or Src (figure 3) (Albrecht *et al.* 1999).

5. GROWTH-TRANSFORMATION OF HUMAN T CELLS

(a) *Scientific background of T-cell transformation*

Rapidly proliferating T-lymphoblastic tumour cell lines are frequently used as a cellular and biochemical model for primary human T cells. This model is limited because tumour-derived cell lines such as Jurkat (Schneider *et al.* 1977) have a strongly altered phenotype in comparison with primary T cells with respect to signal transduction, gene regulation and proliferation control. In contrast, primary T-cell cultures are limited in their natural life span. It is laborious and frequently impossible to grow primary T lymphocytes to large cell numbers, and it requires considerable effort to amplify the T cells in periodic response to a specific antigen in the presence of accessory cells expressing the appropriate MHC restriction elements. The immortalization of functional human T cells should be the ideal way to solve such problems. Virus-mediated transformation is an established routine laboratory method for human B lymphocytes, which are efficiently immortalized *in vitro* by EBV (Henle *et al.* 1967). This method has been instrumental for the analysis of the human B-cell repertoire and function. EBV-transformed lymphoblastoid B cells retain their antigen-presenting capability and are widely used to study T-cellular antigen specificity. Under special culture conditions, EBV was even described to be capable of immortalizing human T cells, although at low efficiency in comparison with human B cells (Groux *et al.* 1997). An established method to transform human T cells in culture uses HTLV-1 (Miyoshi *et al.* 1981; Popovic *et al.* 1983; Yamamoto *et al.* 1982). For numerous applications, this approach proved useful, although the transformation is largely confined to $CD4^+$ T cells. Retrovirus-transformed T lymphocytes produce HTLV-1 virions regularly. However, the HTLV-1-transformed T cells tend to lose their T-cell-receptor complex, their cytotoxic activity and their dependence on IL-2 after prolonged cultivation (Inatsuki *et al.* 1989; Yssel *et al.* 1989).

Another method of T-cell transformation in cell culture became available through the observation that cell-free HVS strain C488 was capable of stimulating human T lymphocytes to stable antigen-independent growth in culture (Biesinger *et al.* 1992). These growth-transformed human T cells did not shed virus particles and retained many essential T-cell functions including the MHC-restricted antigen-specific reactivity of their parental T-cell clones (Bröker *et al.* 1993; De Carli *et al.* 1993; Mittrücker *et al.* 1993; Weber *et al.* 1993). These observations have opened up a novel research direction which links T-cell biology, signal transduction pathways and transforming functions to HVS, an oncogenic herpesvirus which has thus been converted to an immunological tool.

(b) *Infection of human cells by a monkey herpesvirus*

The infection of human cells by HVS is a prerequisite of the growth transformation of human T lymphocytes. The simian HVS is capable of infecting various human cell types in principle. The strain SMHI of HVS subgroup B revealed a weak productive activity in primary human foetal cells (Daniel *et al.* 1976*a*). Selectable HVS recombinants derived from strain A11 were

used to study the spectrum of cells which could be infected by the virus (Simmer *et al.* 1991). A broad range of epithelial, mesenchymal and haematopoietic cell types became persistently infected and carried non-integrated episomal DNA of the recombinant viruses. The pancreatic carcinoma line PANC-1 and human foreskin fibroblasts even produced infectious virus under selection conditions (Simmer *et al.* 1991; Stevenson *et al.* 2000*b*). These findings suggested that the as yet unidentified receptor used by HVS is widely distributed among various tissues. The receptor seems to be well conserved among the species because rabbit T cells can also be infected and transformed by HVS strains (Ablashi *et al.* 1985; Medveczky *et al.* 1989). Cell lines which had been infected with the recombinants under selection pressure retained the non-integrated viral episomes after withdrawal of the selecting drug for long time-periods. The lack of counterselection against cells with persisting viral episomes suggested that the virus persists with suppressed viral gene expression (Simmer *et al.* 1991). This model is also supported by the observations that the persisting non-integrated viral episomes were heavily methylated in simian tumour cells (Desrosiers *et al.* 1979) and may carry extensive genomic rearrangements or deletions (Kaschka-Dierich *et al.* 1982; Schirm *et al.* 1984). These observations were made using mainly HVS strain A11. It is likely that they are also valid for subgroup C strains and for transformed human T cells. Although monkey T lymphocytes produce HVS particles in many cases, it was not possible to isolate virus from transformed human T-cell cultures which carry non-integrated viral episomes in high copy number (Biesinger *et al.* 1992). Even after treatment with phorbol esters, nucleoside analogues and other drugs known to cause reactivation of other viruses such as EBV, or after specific or non-specific stimulation of the T cells, virion production could not be demonstrated (Fickenscher *et al.* 1996*a*). Nevertheless, it will be difficult to provide formal proof that the virus can never be reactivated from transformed human T lymphocytes.

(c) *The preserved functional phenotype of transformed human T cells*

The growth-transformation procedure of human T lymphocytes by HVS C488 (Biesinger *et al.* 1992) has been further described in detailed methodological reviews (Fickenscher & Fleckenstein 1998; Meinl & Fickenscher 2000). The infection of peripheral blood mononuclear cells, cord blood cells, thymocytes or established T-cell clones by HVS C488 results in continuously growing T-cell lines which do not release virus particles and show the morphology of T blasts with irregular shape. Typically, the cell number increases by factors of two to four per week. Optimal growth of HVS-transformed T cells depends on a high cell density and on exogenous IL-2. However, in contrast to primary T cells, regular restimulation with antigen or mitogen in the presence of feeder or antigen-presenting cells is not necessary (Biesinger *et al.* 1992). $CD4^+CD8^-$ or $CD4^-CD8^+$ T cells carrying αβ- or γδ-type T-cell receptors have been transformed by HVS. Mixed populations may occur when polyclonal populations are infected. The transformation of established T-cell clones demonstrated that the phenotype of the parental T cells is conserved (table 3) (Bröker *et al.* 1993; De Carli *et al.* 1993; Fickenscher *et al.* 1997; Klein *et al.* 1996; Pacheco-Castro *et al.* 1996; Weber *et al.* 1993; Yasukawa *et al.* 1995). While initially there is no apparent bias in the range of Vβ family expression in polyclonally derived T-cell lines, the outgrowth of a few clones is commonly observed in long-term culture (Fickenscher *et al.* 1996*b*; Knappe *et al.* 1997). The karyotypes of a series of HVS-transformed T-cell lines were analysed in detail and found to be normal (Troidl *et al.* 1994).

The surface phenotype of the transformed T lymphocytes corresponds to that of mature, activated T cells (Biesinger *et al.* 1992; Fickenscher & Fleckenstein 1998; Meinl *et al.* 1995*a*; Meinl & Fickenscher 2000). The typical surface markers of mature T cells on HVS-transformed T lymphocytes are the T-cell receptor, CD3, CD2, CD4 or CD8, CD5, CD7, CD11a/CD18 (LFA-1, lymphocyte function-associated antigen 1), CD45, CD54 (ICAM-1, intercellular adhesion molecule 1), and CD58 (LFA-3). As typical activation markers, CD25 (IL-2Rα), CD26, CD30, CD40 ligand, CD69, CD86 (B7.2), and MHC class II are detectable on HVS-transformed T cells. The surface antigen CD56 (a natural killer (NK) cell marker) is typically expressed, while the NK markers CD16 and CD57 are lacking. The RO and RB isoforms of the membrane-bound phosphatase CD45, which are typically found on mature memory T cells, were both present on the $CD4^+$-transformed T lymphocytes. The $CD8^+$-transformed cell lines, however, also expressed the CD45 isoform RA, a marker which is considered typical for naive T cells and their precursors. $CD34^+$ triple-negative thymocytes matured to either αβ or γδ T cells after HVS transformation, depending on the cytokines added (Pacheco-Castro *et al.* 1996). Remarkably, the phenotype of HVS-transformed T cells is stable for many months in culture. This is a significant technical advance over other methods of immortalization of human T cells, such as the hybridoma technique or infection with HTLV-1.

Several groups have shown initially that the MHC-restricted antigen-specificity of the T-cell receptor is conserved after transformation. Characterized T-helper cell clones reacting specifically to myelin basic protein (Meinl *et al.* 1995*b*; Weber *et al.* 1993), tetanus toxoid (Bröker *et al.* 1993), nickel ions (Mittrücker *et al.* 1993), bovine 70 kDa heat-shock protein, *Lolium perenne* group I antigen, *Toxocara canis* excretory antigen, and to purified protein derivative from *Mycobacterium tuberculosis* (De Carli *et al.* 1993) were successfully transformed and retained their HLA-restricted antigen specificity. The basal proliferation activity, which is probably due to CD2 contacts with CD58-bearing cells (Mittrücker *et al.* 1992), may interfere with the demonstration of antigen specificity, since the antigen-presenting cells alone cause a stimulation of the transformed T cells. This antigen-independent proliferation of the transformed antigen-specific clones could be reduced by using monoclonal antibodies against CD58 and CD2 together with HLA-transfected mouse L cells as antigen-presenting cells (Weber *et al.* 1993), or by starving the cells prior to antigen presentation (Bröker *et al.* 1993). In all three conditions, clear responses to antigen contact were noticed, as measured by proliferation and cytokine production. Interferon-γ (IFN-γ) production is the best parameter for measuring the activation status of

Table 3. *Maintained and changed functions of C488-transformed human T cells*

	phenotype
maintained functions	surface phenotype (CD3, CD4, CD8, activation markers) MHC-restricted antigen specificity early signal transduction (calcium ion mobilization, tyrosine phosphorylation) IL-2 dependence B-cell help NK-like cytotoxicity karyotype xenogeneic graft-versus-host disease susceptibility to HIV infection
changed functions	CD2 hyperreactivity shift towards Th1 cytokine secretion (IFN-γ) functional expression of Lyn AK155 expression and secretion

HVS-transformed human T cells (Bröker *et al.* 1993; Weber *et al.* 1993). Another study reported that EBV-specific cytotoxic T-lymphocyte lines retained their antigen-specific reactivity after HVS transformation (Berend *et al.* 1993).

Cytokines are produced by transformed human T cells after activation. IL-2 (Mittrücker *et al.* 1992) and IL-3 (De Carli *et al.* 1993) are secreted by the cells in response to mitogenic or antigenic stimuli. Antibodies against CD25 (IL-2Rα) and against IL-2 and IL-3 suppressed the growth rate; both cytokines seem to support autocrine growth. IL-4 and IL-5 are secreted only at low rates by transformed Th2 cells (De Carli *et al.* 1993). Transformed $CD4^+$ T cells secreted IFN-γ, TNF-α, TNF-β, and GM-CSF (granulocyte-macrophage colony stimulating factor) after specific or non-specific stimulation (Bröker *et al.* 1993; De Carli *et al.* 1993; Meinl *et al.* 1995*b*; Weber *et al.* 1993). Both Th1 and Th2 clones were transformed by HVS. The cytokine pattern of the Th1 clones was enhanced, while the profile of Th2 clones switched to a mixed phenotype after transformation (De Carli *et al.* 1993). HVS-transformed T cells are capable of delivering non-specific B-cell help via membrane-bound TNF-α or via CD40 ligand (Del Prete *et al.* 1994; Hess *et al.* 1995; Saha *et al.* 1996).

The virally transformed human T cells show an inducible non-specific cytotoxic activity. When tested on K562 cells, $CD8^+$ lines and to a lesser extent $CD4^+$ cells showed NK-like cytolytic activity (Biesinger *et al.* 1992). The lectin-dependent cytolytic activity of Th1 clones against P815 target cells was enhanced after transformation, while Th2 clones showed this activity only in the transformed state (De Carli *et al.* 1993). The cytotoxic activity of a transformed $\gamma\delta$ T-cell clone on K562 was inducible by stimulation with IL-12 (Klein *et al.* 1996). A comparison of uninfected clones and their herpesvirus-transformed derivatives indicated that the growth transformation by HVS is not based on a resistance to apoptosis (Kraft *et al.* 1998). By strong stimulation, HVS-transformed T cells can be driven into activation-dependent cell death after treatment with phorbol ester or CD3-specific monoclonal antibodies. This form of cell death is apparently not mediated by CD95–CD95 ligand interaction (Bröker *et al.* 1997).

When early signal transduction properties of the transformed cells were compared with those of the uninfected parental cells, no significant differences were encountered (Bröker *et al.* 1993; Mittrücker *et al.* 1993; Tsygankov *et al.* 1992). After stimulation with IL-2, anti-CD3 and/or anti-CD4, similar patterns of tyrosine phosphorylation and calcium mobilization were observed in primary clones or in transformed lines. In contrast, Jurkat cells (Schneider *et al.* 1977) behaved differently (Bröker *et al.* 1993). The HVS-transformed cell lines were strongly stimulated by cell-bound CD58, which is expressed on cells of various origin. This effect was mediated by CD2–CD58 interaction and led to IL-2 production and enhanced proliferation (Mittrücker *et al.* 1992). The functionality of CD3, CD4 and the IL-2 receptor was shown after stimulation by signal transduction parameters, by proliferation, and by IFN-γ production (Bröker *et al.* 1993; Weber *et al.* 1993). The IL-2-dependent proliferation of transformed lymphocytes was strongly inhibited by soluble CD4 antibodies. This effect could be overcome by high doses of IL-2. In parallel, the activity and abundance of the CD4-bound fraction of the tyrosine kinase $p56^{lck}$ was diminished by anti-CD4 treatment (Bröker *et al.* 1994).

(d) *Changed cellular features after T-cell transformation*

Whereas HVS-transformed T lymphocytes retain multiple normal T-cell functions, only a few specific cellular and biochemical features are typically changed in comparison with their parental cells. First, the protein tyrosine kinase $p53/56^{lyn}$, which is usually expressed in B cells but not in T cells, is enzymatically active in HVS-transformed T cells (Fickenscher *et al.* 1997; Wiese *et al.* 1996), as it is in T cells immortalized by HTLV-1 (Yamanashi *et al.* 1989).

Second, HVS shifts the range of cytokines secreted by stimulated T cells towards a Th1 profile: secretion of IL-2 and IFN-γ is increased, and IL-4 and IL-5 production is diminished in comparison with parental cells (De Carli *et al.* 1993). Particularly, the IFN-γ secretion can be stimulated to very high levels (Bröker *et al.* 1993; De Carli *et al.* 1993; Weber *et al.* 1993). In addition, many of these clones secrete large amounts of the CC-chemokines MIP-1α, MIP-1β (macrophage inflammatory protein 1β) and

RANTES, which correlates with their Th1 phenotype (Mackewicz *et al.* 1997; Saha *et al.* 1998; Schrum *et al.* 1996).

Third, the most striking functional alteration induced by HVS in infected human T cells is a hyperresponsiveness to CD2 ligation (Mittrücker *et al.* 1992). CD2 is an abundant surface molecule on T cells, which, when triggered, can transmit mitogenic signals to the cells in a complex fashion. Binding to its ligand CD58, which can be mimicked by antibodies directed against the CD2.1 epitope, is not sufficient for activation of primary T cells, but simultaneous ligation of a different epitope, CD2.2 or CD2R, is necessary (Meuer *et al.* 1984). In contrast, ligation of CD2.1 alone suffices for strong stimulation of HVS-transformed T cells (Mittrücker *et al.* 1992). The degree of the CD2-hyperreactivity depends on the transforming strain of HVS subgroup C (Fickenscher *et al.* 1997). Since the transformed T cells express the CD2 ligand CD58 at high density on their surface, cell contact leads to autostimulation. This is necessary for their growth since, in contrast to the largely autonomous leukaemic T-cell line Jurkat, deprivation of cell contact by limiting dilution immediately halts the growth of HVS-infected human T cells. Sheep erythrocytes, which have a high density of CD58 on their surface, can provide the required stimulus and rescue the proliferation to a high cloning efficiency. This indicates that the hyperresponsiveness to CD2 ligation contributes to the spontaneous proliferation and to the transformed phenotype of HVS-transformed human T cells. However, transformed T cells from a patient lacking CD18/LFA-1 expression could not be stimulated via the CD2 pathway. This suggests that the CD2 hyperreactivity, although typically observed, might not be essential for the HVS transformation (Allende *et al.* 2000).

In order to clone cellular genes that are differentially expressed in HVS-transformed cells, the technique of subtractive cDNA cloning by representational difference analysis was applied (Knappe *et al.* 1997, 2000*b*). By this approach, a novel cellular gene, *ak155*, was identified which is a sequence homologue of cellular IL-10. Specifically HVS-transformed T cells overexpressed cellular *ak155* and secreted the protein into the supernatant. In other T-cell lines and in native peripheral blood cells, but not in B cells, *ak155* was transcribed at low levels. The AK155 protein was shown to form homodimers like IL-10. As a lymphokine, AK155 may contribute to the transformed phenotype of human T cells after infection by HVS (Knappe *et al.* 2000*b*).

(e) *The state of HVS in transformed human T cells*

Although the individual viral genes and functions have already been described above, here we summarize their specific features as these relate to C488-transformed human T cells. Numerous genomic regions of the virus have been used to search for viral transcripts derived from the persisting multicopy episomes in the non-permissive transformed human T cells. With rare exceptions, viral transcription could not be demonstrated from most genomic regions tested. In the left-terminal H- to L-DNA transition region, which is essential for transformation, the viral gene *stpC/tip* was strongly and inducibly transcribed as a bicistronic message (Fickenscher *et al.* 1996*a*; Medveczky *et al.* 1993). Whereas large amounts of StpC were produced (Fickenscher *et al.* 1996*a*), Tip was expressed at very low levels and was detectable only after immunoprecipitation with Lck antibodies with subsequent Lck phosphotransferase assay (Biesinger *et al.* 1995; Lund *et al.* 1997*a*; Wiese *et al.* 1996). StpC and Tip are the sole viral proteins which have been demonstrated in HVS-transformed human T cells.

The non-coding viral U-RNA genes (HSUR, HVS U-RNA) were abundantly expressed in the presence of episomal viral DNA, in a similar way to the EBER RNAs of EBV (EBV-encoded small RNA) (Ensser *et al.* 1999). The deletion of the respective genes did not influence virus replication or T-cell transformation. In addition, after chemical stimulation of transformed human T cells with phorbol ester, the gene *ie14/vsag* was abundantly transcribed for a few hours only (Knappe *et al.* 1997). By this criterion, *ie14/vsag* can be considered the third transformation-associated gene of HVS C488. The mitogenic properties of the secreted protein would fit well into a model of growth transformation caused by a combination of different virus functions. However, the functional importance of this transformation-associated expression is unclear because deletion studies demonstrated that this gene is dispensable for the transformation of human T cells (Knappe *et al.* 1997). A few other virus genes, mainly with regulatory function, were found transcribed, but at extremely low abundance and only after additional T-cell stimulation: the IE gene *ie57*, the early gene *orf50*, and the viral thymidylate synthase gene (Knappe *et al.* 1997; Thurau *et al.* 2000). This and the fact that virus replication could not be induced by various means in C488-transformed human T cells, argues strongly for a block of virus replication downstream of the level of the expression of the regulatory genes *orf50* and *ie57*.

In particular the HVS strain C488 has been used as a tool for the targeted transformation of human T cells (Biesinger *et al.* 1992). In addition, various subgroup C virus strains are able to transform human T cells, but to a varying extent. Virus strain C484 was reported to transform human T cells to short-term, IL-2-independent growth (Medveczky *et al.* 1993). Different subgroup C strains (C488, C484, Cl39) were compared for growth-transformation of human cord blood T cells. The resulting clonal T-cell lines were either $CD4^+$ or $CD8^+$, and expressed either αβ or γδ T-cell receptors. If transformed by the same virus strain, αβ and γδ clones were similar with respect to viral persistence, virus gene expression, proliferation and Th1-type cytokine production. However, major differences were observed in T cells transformed by different subgroup C strains. Strain Cl39 persisted at low copy number as compared with the high copy number of prototype C488. The transformation-associated genes *stpC* and *tip* of strain C488 were strongly induced after T-cell stimulation. The homologous genes of strain Cl39 were only weakly expressed and not induced after activation. After CD2 ligation of the CD2.1 epitope, C488-transformed T cells produced IL-2, whereas Cl39-transformed cells did not. Correspondingly, Cl39-transformed T cells were less sensitive to cyclosporin A. IFN-γ production was induced to a similar extent in both Cl39- and C488-transformed T cells by the CD2 stimulus. Sequence comparison from different subgroup C

strains revealed a variability of the *stpC/tip* promoter region and of the Lck-binding viral protein Tip. Thus, closely related subgroup C strains of HVS can cause major differences in the functional phenotype of growth-transformed human T cells (Fickenscher *et al.* 1997).

(f) *Transformed T-cell lines for studying primary human immunodeficiencies*

The method of T-cell transformation by HVS C488 has also been successfully applied for the analysis of primary human immunodeficiencies, in which specific T-cell functions are disturbed or missing. Surprisingly, T cells with defects in T-cell-receptor-dependent signalling could also be transformed by HVS. The defect of the CD3γ chain did not prevent transformation (Rodriguez-Gallego *et al.* 1996). The transformed CD3γ-deficient cells were used for biochemical studies of the structure of the T-cell receptor complex (Zapata *et al.* 1999). The transformed T cells from a patient with an atypical X-linked severe combined immunodeficiency (SCID) showed a spontaneous partial reversion of the genetic defect affecting the IL-2Rγ chain (Stephan *et al.* 1996). Transformed CD95-deficient T cells of a human SCID patient were useful for studying CD95-independent activation-dependent cell death (Bröker *et al.* 1997). Transformed T-cell lines were established from a series of further patients with genetic T-cell defects involving the IL-12R (Altare *et al.* 1998) and MHC class II (Alvarez-Zapata *et al.* 1998), and with Wiskott–Aldrich syndrome (Gallego *et al.* 1997). HVS-transformed T cells from a patient with leukocyte adhesion deficiency lacking CD18/LFA-1 expression were, surprisingly, not reactive to CD2 stimulation (Allende *et al.* 2000). HVS-transformation seems promising for studying T cells from SCID patients. In many cases, this has been the only way to cultivate and amplify the patients' cells for further research.

(g) *Human immunodeficiency virus infection of transformed human T cells*

HVS-transformed human $CD4^+$ T cells provide a productive lytic system for T-lymphotropic viruses such as HHV-6 (F. Neipel and B. Fleckenstein, unpublished data) and human immunodeficiency virus (HIV) (Nick *et al.* 1993; Vella *et al.* 1997). The prototype viruses HIV-1IIIB and HIV-2ROD replicated rapidly causing cell death within 14 days. Also a poorly replicating HIV-2 strain and primary clinical isolates replicated to high titres. HVS-transformed human $CD4^+$ T cells can be used for poorly growing HIV strains with narrowly restricted host cell range (Nick *et al.* 1993). Moreover, HVS-transformed T cells can be persistently and productively infected with HIV. In comparison with conventional T-cell lines, the down-regulation of surface CD4 molecules is delayed (Vella *et al.* 1997). Similar to cultures of primary peripheral blood cells, HVS-transformed T cells allow the propagation of macrophage-tropic HIV isolates without selecting for subtypes with changed phenotype or cell tropism (Vella *et al.* 1999*a*,*b*). HVS-transformed $CD8^+$ T cells were shown to secrete soluble HIV-inhibiting factors different from the known inhibitory cytokines (Copeland *et al.* 1995, 1996; Lacey *et al.* 1998; Leith *et al.* 1999). Transformed $CD4^+$ and $CD8^+$ T cells produced varying amounts of the cytokines IL-8, IL-10, TNF-α, TNF-β, RANTES, MIP-1α, and MIP-1β (Mackewicz *et al.* 1997; Saha *et al.* 1998). Surprisingly, transformed $CD4^+$ T-cell clones from AIDS patients produced no RANTES and little or no MIP-1α or MIP-1β and were more readily infectable with HIV in comparison with T cells from non-progressors, which produced high amounts of chemokines and were less infectable (Saha *et al.* 1998). HVS-transformed human $CD4^+$ T cells expressing CCR5 and CXCR4 were fully functional as antigen-presenting target cells for HIV-specific, MHC class I-restricted cytotoxic T-cell activity (Bauer *et al.* 1998).

(h) *Transformed human T cells and animal models*

Animal models are valuable for understanding the significance of *in vitro* growth-transformation vis-à-vis neoplastic transformation *in vivo*. When C488-transformed human T cells were tested for tumorigenesis in nude or SCID mice in conventional implantation experiments, tumour formation could not be observed, whereas xenogeneic graft-versus-host disease could be induced just as it could using primary human T cells (Huppes *et al.* 1994).

The behaviour of HVS C488 in various monkey systems is of interest because on the one hand, HVS is a tumour virus of New World monkeys, and on the other hand Old World monkeys such as macaques are the most used animal model for the close-to-human situation. In New World primate T cells, HVS establishes a semi-permissive infection *in vitro* and *in vivo*: the cells do produce virus for long time-periods, and they become transformed as well (summarized in Fickenscher *et al.* 1996*a*). Transcription of IL-2 and activity of IL-4 have been shown from such cultures (Chou *et al.* 1995). Similar to human T cells, the T lymphocytes from macaque monkeys can be growth-transformed by HVS (Akari *et al.* 1996, 1999; Feldmann *et al.* 1997; Knappe *et al.* 2000*a*; Meinl *et al.* 1997). Some researchers observed IL-2 dependence, others IL-2 independence. In many respects, the transformed macaque T cells resembled their human counterparts. The phenotype of activated T cells was preserved, and antigen-specific T-cell lines against myelin basic protein or streptolysin O retained their reactivity after transformation. The MHC class II-expressing transformed cells were able to present the antigen to each other in the absence of autologous presenter cells (Meinl *et al.* 1997). One major difference is the pronounced frequency of double-positive $CD4^+CD8^+$ T cells, which are uncommon in humans. T-cell immunology and T-cell transformation from macaques is greatly hampered by reactivation of the foamy virus with which most rhesus monkeys in primate centres are infected (Feldmann *et al.* 1997; Knappe *et al.* 2000*a*). Initially, HVS-transformed macaque T cells seemed to be non-permissive for HVS, similar to human T cells (Feldmann *et al.* 1997; Meinl *et al.* 1997). However, in contrast to their human counterparts, these cells were shown to shed low amounts of virus particles in many cases, when a more sensitive method including the centrifugation of tissue culture supernatants was applied (Alexander *et al.* 1997; Knappe *et al.* 2000*a*).

In order to study the behaviour *in vivo* in a close-to-human situation, HVS-transformed autologous T cells of macaque monkeys were infused intravenously into the

respective donor. The animals remained healthy, without occurrence of lymphoma or leukaemia for an observation period of more than one year. Over several months virus genomes were detectable in peripheral blood cells and in cultured T cells (Knappe *et al.* 2000*a*). Monkeys which had previously received autologous T-cell transfusions were protected from lymphoma after challenge infection with the wild-type virus C488. In naive control animals, a high-dose intravenous infection rapidly induced pleomorphic peripheral T-cell lymphoma (Alexander *et al.* 1997; Knappe *et al.* 2000*a*). Thus, HVS-transformed T cells were well tolerated after autologous reinfusion. This may form the basis for a new concept of experimental T-cell-mediated adoptive immunotherapy.

6. HVS AS A VECTOR FOR GENE TRANSFER

(a) *Non-transforming HVS strains as an oncogene trap*

Non-transforming and non-oncogenic HVS deletion variants have been used as eukaryotic expression vectors in order to investigate heterologous oncogenes. HVS deletion mutants without the left-terminal transformation-associated L-DNA region neither cause malignant disease in animals, nor transform simian lymphocytes in culture (Desrosiers *et al.* 1984, 1986). Homologous recombination was used for the insertion of transgenes into the virus genome. The transforming functions of HTLV-1 were defined by using an HVS vector containing the HTLV-1 X region or mutants thereof, instead of the homologous transformation-associated genes from HVS A11. These experiments revealed Tax and not Rex as the transforming principle of HTLV-1 for human T cells. Tax-expressing HVS vectors were able to transform primary human T lymphocytes to IL-2-dependent growth. These transformed lymphocytes contained recombinant episomes in high copy number and closely resembled HTLV-1-transformed cells, expressing CD4, MHC class II and CD25 in large amounts (Grassmann *et al.* 1989, 1992; Grassmann & Fleckenstein 1989; Rosin *et al.* 1998; Schmitt *et al.* 1998).

An improved HVS recombination vector system was used for studying c-*fos* function. Overexpression of the proto-oncogene c-*fos* is known to induce transformation in various systems. c-*fos* recombinant HVS-vectors expressed large amounts of the oncoprotein upon persistent infection of human neonatal fibroblasts. However, these primary mesenchymal cells did not show any sign of transformation (Alt *et al.* 1991; Alt & Grassmann 1993). The transforming function of *stpC* for T-cell transformation *in vitro* was successfully substituted by cellular *ras* (Guo *et al.* 1998), by the *K1* gene of KSHV/HHV-8 (Lee *et al.* 1998*a*), or by *R1* of rhesus monkey rhadinovirus (RRV) (Damania *et al.* 1999) in HVS deletion mutants which lacked *stpC*, but still contained the *tip* gene. In KSHV/HHV-8 the gene *K1* is located at the respective transformation-associated left-terminal L-DNA position (figure 2) and interacts with various cellular signalling molecules via its immunoreceptor activation motif and prevents the transport of the B-cell receptor complex to the cell surface (Lee *et al.* 1998*b*; Lee *et al.* 2000). The ability of *K1* to complement an *stpC* deletion mutant of HVS to a transforming and tumorigenic phenotype identified it as an oncogene (Lee *et al.* 1998*a*). In the case of RRV, the oncogene *R1* is situated at the homologous position (Damania *et al.* 1999, 2000). This system of complementing a transformation-deficient HVS strain to a transforming phenotype by heterologous oncogenes is applicable as an oncogene trap in order to identify novel cellular or viral transforming genes.

(b) *HVS vectors for the expression of heterologous genes*

HVS has been further used as a vector for growth hormone, for secreted alkaline phosphatase, and for green fluorescent protein (Desrosiers *et al.* 1985*b*; Duboise *et al.* 1996; Stevenson *et al.* 1999). An early preclinical gene therapy trial was performed with a non-transforming replication-competent HVS vector expressing the bovine growth hormone in genomic intron-containing configuration. Persistently infected simian T cells produced high amounts of the bovine hormone. Experimentally infected New World primates produced circulating bovine growth hormone and later developed a humoral immune response (Desrosiers *et al.* 1985*b*). These observations suggested that persisting HVS vectors could be used to replace missing or defective genes in hereditary genetic disorders.

The original method of generating HVS expression vectors used homologous recombination via a single stretch of viral terminal L-DNA in the recombination construct (Desrosiers *et al.* 1985*b*; Grassmann & Fleckenstein 1989). A more elaborate procedure for the isolation of HVS mutants was developed by the insertion of an autofluorescent reporter gene flanked by single-cutter restriction endonuclease recognition sites into the viral genome instead of the transforming *stpC* gene. Thus, the reporter gene cassette could easily be replaced by other transgenes after simple restriction enzyme digestion and ligation (Duboise *et al.* 1996). This vector has mainly been used for the expression of heterologous oncogenes in order to assay transforming activity in monkey T cells, as mentioned above (§ 6(a)). An alternative approach has been developed by cloning HVS C488 into cosmid vectors. The co-transfection of overlapping cosmids into permissive epithelial OMK cells led to the reconstitution of recombinant replication-competent virus (Ensser *et al.* 1999). This approach is valuable for generating expression vectors for foreign genes, because a contamination with wild-type virus is excluded.

Non-selectable recombinant viruses expressing an autofluorescent protein were able to transduce human haematopoietic progenitor cells in culture, but at low efficiency and with a tendency towards partially differentiated cells (Stevenson *et al.* 1999). Moreover, active HVS replication was observed in certain human cell types (Stevenson *et al.* 1999, 2000*b*), confirming earlier results (Daniel *et al.* 1976*a*; Simmer *et al.* 1991). HVS efficiently infected totipotent mouse embryonic stem (ES) cells under drug selection pressure. The infected ES cells stably maintained the viral episomal genome and could be terminally differentiated into mature haemopoietic cells, while the heterologous gene was rather stably expressed (Stevenson *et al.* 2000*a*). This system may be of particular interest for studying transgene effects during cell differentiation *in vitro* and *in vivo*.

(c) *A concept for episomal HVS vectors for adoptive immunotherapy*

Gene transfer into primary human T cells by transfection or retroviral transduction methods remains difficult and unreliable with respect to long-term transgene expression. The maintained functional phenotype of HVS-transformed T cells suggested the use of HVS as a vector for human T lymphocytes at least for cell culture experiments. The reactivation of recombinant or wild-type virus from transformed human T cells has not been observed, but cannot be formally excluded. The techniques of homologous recombination and cosmid complementation are applied for constructing replication-defective, but transformation-competent deletion variants which preclude reactivation. Furthermore, additional genes are introduced into subgroup C virus strains by these methods in order to express and to study those gene products in human T lymphocytes.

HVS might be useful as a gene vector for targeted amplification of functional human T cells, even for therapeutic applications if a series of biosafety aspects are clarified. By reinfusion of autologous transformed T cells into the donor macaques, an intrinsic oncogenic phenotype could be excluded, because the animals did not develop disease while the infused T cells persisted for extended periods (Knappe *et al.* 2000*a*). In order to improve the biological safety of such vectors, the prodrug activating gene thymidine kinase of herpes simplex virus was inserted into the genome of HVS. Thymidine kinase-expressing transformed T cells were efficiently eliminated in the presence of low concentrations of ganciclovir over an observation period of one year. At any time during the course of a therapeutic application, thymidine kinase-expressing transformed human T cells might be eliminated after administration of ganciclovir. In principle, this function could be useful for the T-cell-dependent immunotherapy of resistant residual leukaemia while avoiding the risk of uncontrolled graft-versus-host disease (Hiller *et al.* 2000). Replication-deficient vector variants are another step towards applicability. The use of HVS vectors for redirecting the antigen specificity of primary human T cells may provide an important tool for experimental cancer therapy.

7. CONCLUSIONS AND PERSPECTIVES

Since the discovery of the oncogenic properties of HVS approximately 30 years ago (Meléndez *et al.* 1969), research on this virus has always concentrated on the acute lymphoma which is induced after experimental infection in various monkey species other than squirrel monkeys. Although it was initially expected that this disease would serve as a tumour model for human EBV disease, it soon became clear that T cells are the major cell type involved, in contrast to B cells in EBV pathogenesis (Wright *et al.* 1976). Thus, the HVS-induced disease remained a general model for tumour development rather than a specific model for a human disease, since a comparable acute peripheral pleomorphic T-cell lymphoma is not known in humans.

More recently, HVS leukaemogenesis has been discussed as a model for tumour induction by KSHV/HHV-8. Although the specific biology of the respective virus-associated tumours (Kaposi's sarcoma versus T-cell lymphoma) is not directly comparable, it is interesting that these viruses have many genomic features in common (Albrecht *et al.* 1992*a*; Russo *et al.* 1996). Since the putative disease-relevant genes are variable between HVS and KSHV/HHV-8, HVS was proposed as a vector for KSHV/HHV-8 genes in order to study their pathogenic potential (Lee *et al.* 1998*a*). The argument in favour of this application is that HVS replicates in permissive epithelial cells, whereas it is difficult to manipulate KSHV/HHV-8 in the absence of a classical permissive system.

Most rhadinoviruses have sequestered a specific set of homologues to cellular genes which is different in different viruses. Whereas functional tests for such genes are not possible in the case of KSHV/HHV-8 in the absence of a replicative system, cell-homologous genes of HVS were shown by deletion mutagenesis to be dispensable for virus replication, T-cell transformation and pathogenicity. This has led to a new hypothesis, which puts the function of the cell-homologous genes closer to the mechanisms of persistence than to transformation or pathogenesis. However, such work will be hampered by the limited availability of sero-negative squirrel monkeys.

The initial stimulus for research in this field was the definition of an oncogenic, transformation-associated region in the HVS genome (Desrosiers *et al.* 1985*a*). Later, the complete genome sequence of the HVS prototype A11, the first sequenced rhadinovirus, (Albrecht *et al.* 1992*a*) facilitated functional studies on individual virus genes. Subsequently, the genome sequences of a series of other rhadinoviruses were completed, including the closely related HVA (Albrecht 2000). Although according to the official classification HVA even comprises two separate virus species (ateline HV-2 and -3), the impression is that HVA resembles an ancient variant of HVS which has collected a smaller set of cell-homologous genes.

A new chapter of HVS research was initiated by the observation that the HVS strain C488 is capable of transforming human T lymphocytes to stable growth in culture (Biesinger *et al.* 1992). Thus, the transforming virus functions became interesting in the context of signal transduction in human T cells, such as the specific interaction between Tip of HVS C488 and the T-cellular tyrosine kinase Lck (Biesinger *et al.* 1995). HVS-transformed human T cells are a promising tool for laboratory studies in T-cell immunology, including inherited and acquired immunodeficiencies. Surprisingly, the transformed human T cells retained many essential features including the MHC-restricted antigen specificity of their non-transformed progenitor clones (Bröker *et al.* 1993; De Carli *et al.* 1993; Mittrücker *et al.* 1993; Weber *et al.* 1993). Because transformed autologous T cells are well tolerated in macaque monkeys (Knappe *et al.* 2000*a*), and because HVS is as an efficient vector for delivering foreign genes into primary human T lymphocytes (Hiller *et al.* 2000), the therapeutic application of HVS as a vector is considerable.

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